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PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter II of the Patent Cooperation Treaty)

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference PCT 0487/RH/sgm	**FOR FURTHER ACTION**	See Form PCT/IPEA/416
International application No. PCT/IN2004/000203	International filing date *(day/month/year)* 09.07.2004	Priority date *(day/month/year)* 09.07.2003
International Patent Classification (IPC) or national classification and IPC C12N9/16		
Applicant INDIAN COUNCIL OF MEDICAL RESEARCH et al.		

1. This report is the international preliminary examination report, established by this International Preliminary Examining Authority under Article 35 and transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 8 sheets, including this cover sheet.
3. This report is also accompanied by ANNEXES, comprising:
   a. ☒ *sent to the applicant and to the International Bureau)* a total of 4 sheets, as follows:
      ☒ sheets of the description, claims and/or drawings which have been amended and are the basis of this report and/or sheets containing rectifications authorized by this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions).
      ☐ sheets which supersede earlier sheets, but which this Authority considers contain an amendment that goes beyond the disclosure in the international application as filed, as indicated in item 4 of Box No. I and the Supplemental Box.
   b. ☐ *(sent to the International Bureau only)* a total of (indicate type and number of electronic carrier(s)) , containing a sequence listing and/or tables related thereto, in computer readable form only, as indicated in the Supplemental Box Relating to Sequence Listing (see Section 802 of the Administrative Instructions).

4. This report contains indications relating to the following items:

☒	Box No. I	Basis of the opinion
☐	Box No. II	Priority
☐	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☒	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☐	Box No. VI	Certain documents cited
☐	Box No. VII	Certain defects in the international application
☒	Box No. VIII	Certain observations on the international application

Date of submission of the demand 08.02.2005	Date of completion of this report 11.08.2005
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized Officer Kalsner, I Telephone No. +49 89 2399-8708

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/IN2004/000203

Box No. I Basis of the report

1. With regard to the **language**, this report is based on the international application in the language in which it was filed, unless otherwise indicated under this item.

   ☐ This report is based on translations from the original language into the following language , which is the language of a translation furnished for the purposes of:
   - ☐ international search (under Rules 12.3 and 23.1(b))
   - ☐ publication of the international application (under Rule 12.4)
   - ☐ international preliminary examination (under Rules 55.2 and/or 55.3)

2. With regard to the **elements*** of the international application, this report is based on *(replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report)*:

Description, Pages

1-35 as originally filed

Sequence listings part of the description, Pages

1-26 as originally filed

Claims, Numbers

1-28 filed with telefax on 15.07.2005

Drawings, Sheets

1/12-12/12 as originally filed

☒ a sequence listing and/or any related table(s) - see Supplemental Box Relating to Sequence Listing

3. ☐ The amendments have resulted in the cancellation of:
   - ☐ the description, pages
   - ☐ the claims, Nos.
   - ☐ the drawings, sheets/figs
   - ☐ the sequence listing *(specify)*:
   - ☐ any table(s) related to sequence listing *(specify)*:

4. ☐ This report has been established as if (some of) the amendments annexed to this report and listed below had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).
   - ☐ the description, pages
   - ☐ the claims, Nos.
   - ☐ the drawings, sheets/figs
   - ☐ the sequence listing *(specify)*:
   - ☐ any table(s) related to sequence listing *(specify)*:

* *If item 4 applies, some or all of these sheets may be marked "superseded."*

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Box No. IV Lack of unity of invention

1. ☐ In response to the invitation to restrict or pay additional fees, the applicant has:
   - ☐ restricted the claims.
   - ☐ paid additional fees.
   - ☐ paid additional fees under protest.
   - ☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied with and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is
   - ☐ complied with.
   - ☐ not complied with for the following reasons:

4. Consequently, this report has been established in respect of the following parts of the international application:
   - ☒ all parts.
   - ☐ the parts relating to claims Nos. .

Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-28
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-28
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-28
	No:	Claims	

2. Citations and explanations (Rule 70.7):

see separate sheet

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

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Supplemental Box relating to Sequence Listing

Continuation of Box I, item 2:

1. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application and necessary to the claimed invention, this report has been established on the basis of:

   a. type of material:

   ☒ a sequence listing

   ☐ table(s) related to the sequence listing

   b. format of material:

   ☒ in written format

   ☒ in computer readable form

   c. time of filing/furnishing:

   ☒ contained in the international application as filed

   ☐ filed together with the international application in computer readable form

   ☒ furnished subsequently to this Authority for the purposes of search and/or examination

   ☒ received by this Authority as an amendment on

2. ☒ In addition, in the case that more than one version or copy of a sequence listing and/or table(s) relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional observations, if necessary:

Ad Section IV: Lack of unity of invention

The present application does not comply with the requirement of unity as set forth in Art. 34(3) and Rule 13 PCT.

An international application must relate to one invention only or to a group of inventions so linked as to form a single general inventive concept.

Unity of invention is fulfilled only when there is a technical relationship among the inventions involving one or more of the same special technical features, special technical features being such features that define a contribution which each of the claimed inventions, considered as a whole, makes over the prior art.

The following two inventions have been identified:

Invention 1: Claims 3, 4, 11, 27 completely, and claims 1, 2, 7-10, 13-26 partially: a Mycobacterium strain with a modified tyrosine phosphatase gene, a recombinant vector, an isolated nucleic acid sequence a method for developing a Mycobacterium strain with a modified tyrosine phosphatase gene; all with respect to mptpA (SEQ ID NO: 11)

Invention 2: Claims 5, 6, 12, 28 completely, and claims 1, 2, 7-10, 13-26 partially: a Mycobacterium strain with a modified tyrosine phosphatase gene, a recombinant vector, an isolated nucleic acid sequence a method for developing a Mycobacterium strain with a modified tyrosine phosphatase gene; all with respect to mptpB (SEQ ID NO: 12)

The technical relationship linking together the different nucleotide sequences can be seen in the fact that they are both encoding a tyrosine phosphatase from M. tuberculosis. As tyrosine phosphatases from Mycobacterium have already been disclosed in the prior art (Koul et al, 2000; WO 0181422) this relationship can no longer be considered novel or inventive. This concept/relationship, therefore, cannot be accepted to constitute a special technical feature as defined above as it does not define a contribution which each of the different claimed inventions, considered as a

whole, makes over the prior art.

Thus, the presently claimed subject-matter falls apart in the above groups of inventions which are not unitarian.

As search and examination of the present application can be carried out without undue effort, the applicant has not been invited, according to Rule 68.1 PCT, to restrict or pay additional examination fees.

Ad Section V: Reasoned statement with regard to novelty, inventive step or industrial applicability

1) Amendments

The amendments filed with the letter dated 21 July 2005 are allowable under Art. 34(2)(b) PCT.

2) Documents

D1...Koul et al. (2000) J. Bacteriology 182: 5425-5432
D2...WO 01 81422

D1 discloses the characterisation of two tyrosine phosphatases isolated from Mycobacterium tuberculosis. It could be shown that the activity of the enzyme could be inhibited by replacing the Cys residues in the active domain of the enzymes (Cys-11 of MptpA and Cys-160 of MptpB) by Ser.

3) Novelty and inventive step

The present application relates to a Mycobacterium strain with a modified tyrosine phosphatase wherein the Mycobacterium strain is not capable of expressing an active tyrosine phosphatase gene and to a method for developing such Mycobacterium strain. Modification is done by replacing part of the gene expressing tyrosine phosphatase by a gene encoding antibiotic resistance.

Claim 1 is directed to a mutant strain of Mycobacterium comprising in its genome a modified tyrosine phosphatase gene selected from mptpA bearing SEQ ID NO: 15 and mptpB bearing SEQ ID NO: 16, the strain being incapable of expressing active tyrosine phosphatase.

None of the available prior art discloses a Mycobacterium strain comprising the sequences as specified in claim 1. While the modification of mptpA or mptpB gene of Mycobacterium tuberculosis has been disclosed in D1 it is not disclosed or suggested in the prior art to replace part of the nucleic acid sequence coding for tyrosine phosphatase by an antibiotic resistance marker.

Claim 1 and claims directly or indirectly dependent thereon (i.e. claims 2-28) are therefore considered to meet the requirements of Art. 33(2)(3) PCT.

Ad Section VIII: Certain observations on the international application

1) **Claims 4, 6, 22 and 23** do not meet the requirements of Art. 6 PCT as they refer to a vector by arbitrary designation. Claims, however, have to be defined by technical (= structural) features.

2) **Claims 7-10, 20 and 25** do not meet the requirements of Art. 6 PCT for the following reasons:

According to the description SEQ ID NO 15 and 16 comprise the coding sequences of tyrosine phosphatase which are disrupted by insertion of a hygromycin resistance marker gene.

Claims 7 and 8 refer to this marker gene in broader terms. It is not clear how the sequence as specified by SEQ ID NO: 15 or 16 could possibly encompass resistance to other antibiotics than hygromycin.

The same arguments hold for **claims 9 and 10** which further define the second antibiotic resistance gene and which (among others) refer back to claims 4 and 6. From the description it can be derived that the vectors pAKΔA and pBKΔB carry an

additional antibiotic resistance marker for kanamycin. The dependency of these claims is thus unclear.

We claim:

1. A mutant strain of mycobacterium comprising in its genome a modified tyrosine phosphatase gene selected from *mptpA* bearing SEQ ID NO.15 and *mptpB* bearing SEQ ID NO.16, the strain being incapable of expressing active tyrosine phosphatase.
2. A strain as claimed in claim 1, wherein the mycobacterium strain is selected from a group consisting of *M. tuberculosis* and *M. bovis*.
3. A recombinant vector comprising a modified *mptpA* gene bearing SEQ ID NO.15.
4. A vector as claimed in claim 3, wherein the vector is pAK A.
5. A recombinant vector comprising a modified *mptpB* gene bearing SEQ ID NO.15.
6. A recombinant vector as claimed in claim 5, wherein the vector is pBk B.
7. A recombinant vector as claimed in any of claims 3-6, wherein the modified *mptpA* or *mptpB* gene includes an internal region substituted with a first antibiotic resistance marker gene.
8. A recombinant vector as claimed in claim 7, wherein the antibiotic resistance marker gene imparts resistance to an antibiotic selected from hygromycin or chloramphenicol, preferably hygromycin.
9. A recombinant vector as claimed in any of claims 3-6, further comprising a second antibiotic marker gene inserted in its backbone.
10. A recombinant vector as claimed in claim 9, wherein the second antibiotic marker gene imparts resistance to an antibiotic selected from kanamycin or gentamycin.

11. An isolated nucleotide sequence bearing SEQ ID NO.15 and representing modified *mptpA* gene.
12. An isolated nucleotide sequence SEQ ID No.16 and representing modified *mptpB* gene.
13. A method for developing a mutant mycobacterium strain comprising a modified tyrosine phosphatase gene in its genome, comprising the following steps:
    a. extracting genomic DNA from a mycobacterium strain,
    b. amplifying a tyrosine phosphatase gene alongwith flanking sequences using a primer designed from the genomic DNA of step (a) to obtain a DNA fragment,
    c. characterizing the fragment of step (b) by sequencing and restriction enzymatic analysis,
    d. cloning the fragment of step (b) in a non-replicative vector,
    e. modifying the fragment in the non-replicative vector of step (d) by performing a step selected from insertion, deletion mutation or substitution,
    f. inserting a first antibiotic resistance marker gene within the fragment of step (e) to obtain a non-replicative vector comprising a modified tyrosine phosphatase gene selected from *mptpA* bearing SEQ ID 15 or *mptpB* bearing SEQ ID 16,
    g. cloning of a second antibiotic resistance marker gene in the backbone of the non-replicative vector of step (f), to obtain a recombinant vector,
    h. introducing the recombinant vector of step (g) to obtain into a mycobacterium strain,

i. selecting for primary recombinant mycobacterium strains using the first antibiotic resistance marker gene,

j. culturing the primary recombinant mycobacterium strain of step (i) harboring the first antibiotic resistance marker gene,

k. selecting for secondary recombinant mycobacterium strains of step (j) that are sensitive to the second antibiotic resistance gene present in the vector backbone,

l. culturing the secondary recombinant mycobacterium strains of step (k), to obtain a recombinant mycobacterium strain harboring the modified tyrosine phosphatase gene which shows defective growth in activated macrophages and animals.

14. A method as claimed in claim 13, wherein the mycobacterium species is selected from a group consisting of *M. tuberculosis* and *M. bovis*.

15. A method as claimed in claim 13, wherein, the primer designed in step (b) is selected from any of SEQ ID NO: 1 to 4 for amplification of *mptpA* alongwith its flanking regions and any of SEQ ID NO: 5 to 8 for amplification of *mptpB* alongwith its flanking regions.

16. A method as claimed in claim 13, wherein the tyrosine phosphatase gene is *mptpA* gene of SEQ ID No. 11

17. A method as claimed in claim 13, wherein the tyrosine phosphatase gene is *mptpB* gene of SEQ ID No. 12.

18. A method as claimed in claim 13, wherein in step (b) the DNA fragment is a sequence bearing SEQ ID No. 13.

19. A method as claimed in claim 13, wherein in step (b) the DNA fragment is a sequence bearing SEQ ID No. 14.
20. A method as claimed in claim 13, wherein the first antibiotic resistance marker gene imparts resistance to an antibiotic selected from hygromycin or chloramphenicol, preferably hygromycin.
21. A method as claimed in claim 13, wherein the second antibiotic marker gene imparts resistance to the antibiotic kanamycin.
22. A method as claimed in claim 13, wherein in the recombinant vector is pAK A.
23. A method as claimed in claim 13, wherein in the recombinant vector is pBk B.
24. A method as claimed in claim 13, wherein the vector is introduced by electroporation or through phages.
25. A method as claimed in claim 13, wherein primary recombinant mycobacterium strain is selected by using an antibiotic selected from hygromycin or chloramphenicol.
26. A method as claimed in claim 13, wherein in step (k) the secondary recombinant mycobacterium strain is resistant to hygromycin or chloramphenicol but sensitive to the second antibiotic kanamycin.
27. A primer sequence adapted for amplification of *mptpA* gene selected from any of SEQ ID No. 1 to 4 alongwith its flanking regions.
28. A primer sequence adapted for amplification of *mptpB* gene selected from any of SEQ ID No. 5 to 8 alongwith its flanking regions.